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Pull-down of metalloproteins in their native states using desthiobiotin-based probes

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Abstract

One-third of all proteins are estimated to require metals for structural stability and/or catalytic activity. Desthiobiotin probes containing metal binding groups can be used to capture metalloproteins with exposed active-site metals under mild conditions to prevent changes in metallation state. The proof-of-concept was demonstrated with carbonic anhydrase (CA), an open active site, Zn-containing protein. CA was targeted using sulfonamide derivatives. Linkers of various lengths and structures were screened to determine the optimal structure for capture of the native protein. The optimized probes could selectively pull down CA from red blood cell (RBC) lysate and other protein mixtures. Pull-down of differently metallated CAs was also investigated.

Graphical Abstract

Desthiobiotin probes with sulfonamide groups enable pull-down of carbonic anhydrase without destruction of protein metallation state.

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Keywords

metalloproteins; carbonic anhydrase; desthiobiotin; sulfonamide; proteomics

Metalloproteins comprise ~30% of the proteome^[1] and contain metal co-factors that serve as structural components and/or as sites for catalysis. These metalloproteins can exist in several states including *apo* (metal-free), *holo* (metal-bound), and mismetallated (not fully metallated, incorrect metal in binding site, etc.) depending on the cellular context. The metal requirements for most metalloproteins do not match their metal binding preferences and without cellular regulation, these proteins tend to bind divalent transition metal ions following the Irving-Williams series. For approximately 30% of metalloproteins metallation is achieved with the help of metal delivery systems.^[2] The remaining 70% acquire the required metal ions from buffered metal pools where concentrations of competitive metals are restricted.^[2a] Nevertheless, mismetallation can occur under certain conditions associated with oxidative stress or environmental contamination.^[3] Mismetallation can lead to protein misfolding and aggregation, which is often associated with disease states.^[4] Therefore, designing new probes that will enable identification of cellular metallation status is essential for addressing questions of both metal ion homeostasis and dyshomeostasis.

Previous efforts in metalloproteomics have largely relied on a combination of chromatographic protein separation, elemental detection (e.g. inductively couple plasma mass spectrometry), and mass spectrometry-based protein identification.^[5] While powerful, a caveat of these techniques is that proteins at lower abundance are often not detected due to detection limits and challenges with separation. Traditional pull-down techniques are often inadequate for studies of non-covalent cofactors like metals. Because these interactions are non-covalent, they can be easily destroyed through the use of strong acids/bases, concentrated inorganic salts, strong complexing anions, and heat, which leads to loss or exchange of metal cofactors and/or denaturation of proteins.^[6] In recent years, activity-based protein profiling strategies have been developed to capture a number of enzymes including metalloenzymes.^[7] These tools have proven powerful in a number of systems, though the methods often incorporate tryptic digest and other procedures that could destroy native protein structure and prevent identification of the cellular metallation state of a metalloenzyme.

Herein, we present a method for capturing metalloenzymes that enables recovery of enzymes in their native metallation state using targeted small molecule probes. These probes contain a metal-binding group (MBG), a biotin analogue with reduced streptavidin affinity, and an appropriate linker that allows formation of a ternary complex between the intact target protein, streptavidin, and probe (Fig. 1). When incubated with a protein mixture, the probe can bind to metalloenzymes *via* coordination of the MBG to metal ions. The metalloenzyme-probe complexes are then captured by magnetic streptavidin beads, allowing for their separation from proteins that cannot interact with the MBG. Enrichment of metalloproteins is achieved by eluting the metalloprotein-probe complexes anchored on the surface of streptavidin beads with biotin. Since the low-affinity biotin-based group on the

probe cannot compete with the parent biotin, this elution can be done under mild, non-denaturing conditions, which is crucial to maintaining native metallation states.

We selected carbonic anhydrase (CA) as a proof-of-concept system for this strategy because this protein, as well as its inhibitors, have been widely studied.^[8] CA is a Zn^{2+} -dependent enzyme that catalyzes the reversible conversion between carbon dioxide and water and bicarbonate. Most well-known inhibitors of CA are based on sulfonamides, so this group was employed as the MBG in the probe design.^[9] Sulfonamide binds to CA via coordination of the deprotonated sulfonamide nitrogen to the active site Zn and stabilized by two hydrogen bonds of the sulfonamide group to residue Thr199, which is present in all isoforms.^[10] Most importantly, sulfonamides are not known to strip Zn^{2+} from the CA active site. Linked to the sulfonamide is a desthiobiotin moiety, which binds to streptavidin with the same specificity as biotin, but can dissociate more easily due to its lower affinity for the protein ($K_d = 10^{-11}$ vs 10^{-15} M for biotin).^[11]

To arrive at a structurally optimized and functional probe that could be used for pull-down experiments, several probes with different linker lengths and compositions were synthesized (Fig. 2). The two sulfonamide moieties that were included in this study were benzenesulfonamide (probes **1**, **2a**, **2b**, and **3**) and benzothiazole sulfonamide (probe **4**). Benzenesulfonamide is a very common CA binding motif which has been included in the designs of several novel CA inhibitors.^[9] The benzothiazole motif can be seen in ethoxzolamide, a drug which was once in clinical use and has significantly tighter binding with many CA isoforms compared to benzenesulfonamides.^[12] Detailed syntheses for all probes are reported in the Supporting Information. The main strategy involved the coupling of desthiobiotin-NHS ester and an appropriate aryl sulfonamide-functionalized chain containing a terminal NH_2 group.

As shown in the proposed workflow (Fig. 1), successful protein enrichment is dependent on many factors, with one of those being the formation of the ternary streptavidin-probe-CA complex. A good probe is one that allows sufficient ternary complex formation. For these studies, we used avidin as a surrogate for streptavidin; avidin is a heavily glycosylated version of streptavidin and interacts with desthiobiotin the same way streptavidin does.^[13] The extent of ternary complex formation can be estimated by a *p*-nitrophenylacetate (NPA) assay, which relies on the hydrolysis of NPA catalyzed by CA. We performed these studies with bovine carbonic anhydrase (bCA). When uninhibited, bCA quickly consumes the colorless NPA and yields the yellow-colored *p*-nitrophenolate ion, resulting in a rapid increase of absorbance at 400 nm.^[14] Conversely, sulfonamide-inhibited bCA will show a slower rate of increase or absence of the absorbance at 400 nm.

The extent of avidin-probe-bCA formation could be derived from this assay by looking at the NPA hydrolysis rate of each probe in the presence of both avidin and bCA (Fig. 3). In the absence of avidin, all probes could inhibit bCA moderately to very strongly. Probe **1**, a probe with a pure alkyl linker, inhibited CA more strongly than probes **2a** and **2b** which had longer linkers comprised of ethylene glycol units. The highly hydrophilic ethylene glycol linkers likely attenuated the interaction between probes **2a** and **2b** and the hydrophobic pocket of CA. The inhibition studies are consistent with the measured dissociation constants

of the probes with bCA, as determined by a competition assay with a fluorescent inhibitor of CA (**1**: 7.2×10^{-9} M, **2a**: 7.8×10^{-7} M, **2b**: 9.9×10^{-7} M, **3**: 1.1×10^{-8} M, **4**: 3×10^{-9} M) (Fig. S1). Probes **1**, **2a**, and **2b**, however, could not inhibit CA activity in the presence of avidin as NPA hydrolysis activity was high in these samples. The inability of a probe to inhibit CA in the presence of avidin suggested that the avidin-probe complex could not bind to CA, which ultimately meant that there was insufficient formation of the ternary complex. Only probes **3** and **4** displayed excellent CA inhibition in the presence of avidin (Fig. 3), indicated by the lack of NPA hydrolysis activity. The linker in probes **3** and **4** was long enough to allow ternary complex formation and additionally contained hydrophobic portions to facilitate strong CA interactions. The probe **4**-bCA complex was observed using native mass spectrometry with the Zn^{2+} present in the active site (Fig. S18). Apo bCA was not observed; suggesting that probe **4** does not remove the native Zn^{2+} ion from the protein.

A native PAGE experiment was carried out with probes **3** and **4** to further confirm their ability to form the ternary avidin-probe-bCA complex (Fig. 4). In this experiment, the electrophoresis was run with a reverse electrode set-up to allow migration of avidin ($\text{pI} = 10$) and the ternary complex into the gel. Free CA ($\text{pI} = 5.4$) will not migrate into the gel under these conditions and thus will not be observed. Since avidin has four binding sites for desthiobiotin, two different equivalents of probes were tested to see if there was a difference in avidin mobility at half occupancy and full occupancy. In the absence of CA, 2 equiv and 5 equiv of probe did not change how avidin moved on the gel (lanes 2, 4, 6, 8). The difference of more than 2 kDa at max occupancy of avidin was not enough to affect protein migration. However, upon addition of CA, changes in band migration were observed. The new band did not move as far as avidin (lanes 3, 5, 7, 9), which is consistent with formation of a species with a molecular weight significantly higher than avidin, a lower pI , or both. We presume this species to be the ternary complex of avidin-probe-CA.

The protein substrate scope of sulfonamide probes was investigated by using probe **4** to pull down bCA and human carbonic anhydrase II (hCAII) from two mixtures containing a variety of proteins (Fig. 5B). These proteins include bovine serum albumin (BSA), lysozyme, and several metalloproteins, including catalase, New Delhi metallo- β -lactamase 1 (NDM1), superoxide dismutase 1 (SOD1), myoglobin (Mb), and hemoglobin (Hb). Probe **4** showed exclusive selectivity for pull-down of bCA and hCAII. It did not interact with BSA, lysozyme, or heme proteins. Importantly, other Zn-containing enzymes (NDM1 and SOD1) were also not captured.

Probe **4** was then used to pull down CA from a red blood cells (RBC) lysate (Fig. 5C). The RBC lysate was prepared using a mixture of chloroform and ethanol according to literature procedures; this process significantly reduces the Hb concentration in these samples.^[15] Under these conditions, the aqueous supernatant lysate should contain bCA, Hb, oxidases, and a small amount of catalase. The total protein concentration was estimated to be 20 mg/mL. In the pull-down experiment, probe **4** showed very good bCA enrichment in this mixture of cellular proteins.

Probe **4** can further be used to pull down CA in different metallation states including Mn^{2+} -CA,^[16] Fe^{3+} -CA,^[17] Co^{2+} -CA,^[16a, 17–18] Ni^{2+} -CA,^[16a, 17] Cd^{2+} -CA,^[17] Cu^{2+} -CA,^[16a] and

bis-Cu²⁺-CA,^[16a, 19] which has Cu²⁺ bound to the active site as well as to a secondary site. Of these, Mn²⁺-, Fe³⁺-, and Co²⁺-CAs were successfully captured (Fig. 5). The pull-down was especially effective for Co²⁺-CA, which can be explained by the ionic radius and preferred geometry of Co²⁺. It has the same ionic radius as Zn²⁺ (0.74 Å), and also has a propensity to form tetrahedral coordination complexes.^[16a] The similarities preserve active site interactions with the sulfonamide motif in probe **4**. Further, Co²⁺-CA is the only non-zinc CA that has comparable esterase activity to Zn²⁺-CA's.^[17] Although Mn²⁺ also can adopt tetrahedral coordination, its slightly larger size can distort the CA active site to some extent, which explains why Mn²⁺-CA was not isolated in great amount. The pull-down of Fe³⁺-CA is interesting as not much is known about this form of CA apart from the fact that it does not exhibit esterase activity.^[17] Ni²⁺, Cd²⁺, Cu²⁺, and bis-Cu²⁺-CAs were not captured. The results can be explained by active site distortion due to large ionic size, in the case of Cd²⁺, or the non-tetrahedral coordination geometry at the active site in the case of Ni²⁺ and Cu²⁺.^[17] The relative levels of protein captured correlate with the esterase activity of the different metallated forms of CA, with Co²⁺-CA having very high activity, Mn²⁺-CA and Fe³⁺-CA showing low levels of activity, and the other forms showing no activity. Thus, probe **4** demonstrates the ability to capture CA protein capable of catalytic activity.^[17]

In summary, the optimized desthiobiotin-sulfonamide probe **4** could capture CAs from different protein mixtures under very mild, non-denaturing conditions. Pull-down of some of the CAs with alternate metallation states (Mn²⁺, Fe³⁺, and Co²⁺) was also possible, suggesting that this probe design can be used to study protein mismetallation in cells. These results demonstrate the potential of our proposed workflow for metalloproteomic purposes. Varying the MBG on the probe structure will allow capture of various classes of metalloproteins with exposed metal sites. Future efforts include enhancing the current design though inclusion of longer linkers and covalent crosslinking groups.

Experimental Section

Native PAGE to confirm ternary complex formation:

Probes **3** and **4** (10 mM in DMSO) were used in this experiment. Avidin and bCA were prepared as 10 mg/mL solutions in 50 mM HEPES, 0.1 M KNO₃, pH 7.2. The samples were allowed to incubate for 10 minutes before loaded into 7.5% Mini-Protean TGX gel. The gel was run with reverse electrode at 100V at 4°C for 1.5 h in a non-SDS Tris-glycine buffer (25 mM Tris base, 250 mM glycine).

Protein pull-down and SDS-PAGE to determine protein substrate scope of probe **4**:

Two protein mixtures (60 µL) containing NDM1, SOD1, Mb, Hb, catalase, BSA, lysozyme (~10 µg of each protein) were prepared using PBS buffer, pH 7.4. hCA II (~10 µg) was added to one sample and bCA (~10 µg) was added to the other. Probe **4** solution (10 mM, 0.25 µL) was added to the mixtures and the mixtures were incubated for 20 minutes. Simultaneously, streptavidin beads (10 mg/mL, 100 µL) were washed with PBS buffer (2 × 400 µL). The protein mixture was then added to the washed beads and incubated for 1 hour. The magnetic beads were separated from the supernatant by a strong magnet. The supernatant was removed and the beads were washed with PBS buffer (2 × 200 µL). Biotin

solution (10 mM in PBS, 80 μ L) and MeOH (20 μ L) were added to the beads to remove the enriched protein from the bead surface. SDS-PAGE was run at 180 V for 30 min in 1 $\times$ SDS-PAGE buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

Protein pull-down with non-Zn CAs:

Mn²⁺-CA, Fe³⁺-CA, Co²⁺-CA, Ni²⁺-CA, Cd²⁺-CA, Cu²⁺-CA, and bis-Cu²⁺-CA samples (~9 μ g in 50 μ L PBS buffer, pH 7.4) were prepared as described above. Probe 4 solution (10 mM, 0.25 μ L) was added to the mixtures and the mixtures were incubated for 20 minutes. Simultaneously, streptavidin beads (10 mg/mL, 100 μ L) were washed with PBS buffer (2 $\times$ 400 μ L). The protein mixture was then added to the washed beads and incubated for 1 hour. The magnetic beads were separated from the supernatant by a strong magnet. The supernatant was removed and the beads were washed with PBS buffer (2 $\times$ 200 μ L). Biotin solution (10 mM in PBS, 80 μ L) and MeOH (20 μ L) were added to the beads to remove the enriched protein from the bead surface. SDS-PAGE was run at 180 V for 30 min in 1 $\times$ SDS-PAGE buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

Protein pull-down with RBC lysate:

Probe solution (10 mM, 0.25 μ L) was added to ~50 μ L of the above RBC lysate. The mixture was incubated for 20 minutes. Simultaneously, streptavidin beads (10 mg/mL, 100 μ L) were washed with PBS buffer (2 $\times$ 400 μ L). The protein mixture was then added to the washed beads and incubated for 1 hour. The magnetic beads were separated from the supernatant by a strong magnet. The supernatant was removed and the beads were washed with PBS buffer (2 $\times$ 200 μ L). Biotin solution (10 mM in PBS, 80 μ L) and MeOH (20 μ L) were added to the beads to remove the enriched protein from the bead surface. SDS-PAGE was run at 180 V for 30 min in 1 $\times$ SDS-PAGE buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Proposed workflow for enrichment of metalloenzymes using mild conditions to maintain native protein states.

Figure 2.
Structures of desthiobiotin-sulfonamide probes.

Figure 3.

Normalized rate of NPA hydrolysis by bCA incubated with different probes in the absence or presence of avidin. Formation of the *p*-nitrophenolate ion results in an increase in absorbance at 400 nm. Rates of change in absorbance were calculated and normalized to the rate of NPA hydrolysis of the non-inhibited bCA control.

Figure 4. Native PAGE analysis of the ternary complexes avidin-probe **3**-bCA and avidin-probe **4**-bCA (7.5% Mini-Protean TGX gel, reverse electrode, 100V, 4°C, 90 min). Avidin (~67 kD). Avidin-probe (~67 kD). Avidin-probe-bCA (~96 kD).

Figure 5.

(A) SDS-PAGE analysis of the pull-down of differently metallated CAs (4-20% Mini-Protean TGX gel, 180V). Lane 1: MW ladder. Lane 2: commercial Zn bCA (~29 kD). Lanes 3-9: protein pull-down samples. (B) Protein substrate scope of probe **4** was assessed by SDS-PAGE (4-20% Mini-Protean TGX gel, 180V). BSA (~66kD), catalase (monomer, ~60 kD), bCA (~29 kD), hCA II (~29 kD), NDM1 (~28 kD), SOD1 (~16 kD), Mb (~17 kD), Hb (monomer, ~14 kD), lysozyme (~14 kD). Lane 1: MW ladder. Lane 2: Protein sample contained all of the proteins above except hCA II. Lane 3: Pull-down of lane 2 sample. Lane 4: bCA. Lane 5: Protein sample contained all of the proteins above except bCA. Lane 6: Pull-down of lane 5 sample. Lane 7: hCA II. Lane 8: BSA, Mb, and lysozyme. Lane 9: catalase, NDM1, SOD1, and Hb. Lane 10: MW ladder. (C) SDS-PAGE analysis of bovine RBC lysate pull-down (4-20% Mini-Protean TGX gel, 180V). bCA (~29 kD), Hb alpha unit (~14 kD). Protein concentration of the RBC lysate was approximately 20 mg/mL.